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Dissociation of Immunologic and Virologic Responses to Highly Active Antiretroviral Therapy

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Objective: Immunologic markers, levels of HIV DNA, and infectious HIV were compared in partial responders (PR) to HAART who had high plasma HIV RNA levels but stable or increasing levels of $CD4^+$ peripheral blood mononuclear cells (PBMC), and patients with complete failure (CF) who had very low or decreasing levels of $CD4^+$ PBMC and high plasma HIV RNA levels.

Design and Methods: CD4 and CD8 levels were monitored by flow cytometry. β_2-microglobulin (β_2M) and neopterin levels were measured by quantitative enzyme immunoassays. Plasma and PBMC from 11 PR and 13 CF were analyzed for infectious HIV levels in limiting dilution cultures. Polymerase chain reaction (PCR) assays were used to quantify cellular HIV DNA and plasma HIV RNA.

Results: In comparison with CF, PR had little or no $CD4^+$ cell loss, a substantial increase in $CD8^+$ cells, significantly fewer positive plasma HIV cultures ($p = .03$), lower frequencies of infectious HIV in total PBMC ($p = .005$) and in $CD4^+$ PBMC ($p < .001$), and lower frequencies of HIV DNA in $CD4^+$ PBMC ($p = .007$).

Conclusions: Lower levels of infectious HIV and a lower frequency of $CD4^+$ PBMC that contain "productive" HIV DNA in PR as compared with CF may contribute to the stable or increasing $CD4^+$ PBMC levels of the PR. However, HAART may also have effects on lymphocyte homeostasis independent of its antiviral activity.

Key Words: HAART—Immunologic response—Virologic response—HIV.

Highly active antiretroviral therapy (HAART), which includes at least one protease inhibitor (PI) with two reverse-transcriptase inhibitors (RTI), has significantly reduced morbidity and mortality rates in HIV-positive patients (1,2). Many patients experience both immunologic and virologic responses to HAART in terms of increased levels of $CD4^+$ peripheral blood mononuclear cells (PBMCs) and reduced levels of plasma HIV RNA, respectively (3–5). Between 7% and 15% of HAART patients, however, have a seemingly paradoxic response to HAART in that their $CD4^+$ PBMC levels increase substantially but their levels of plasma HIV RNA remain high (6–11). In this report, we classify these patients as partial responders (PR) to HAART and contrast them with those with complete failure (CF) who have neither a significant rise in $CD4^+$ lymphocytes nor a fall in their plasma HIV RNA levels. Plasma HIV RNA levels are a strong predictor of progression to AIDS (12,13) and it has been suggested that these paradoxic responses are transient and may be explained by the rate of disease progression before treatment (10). The levels of infectious HIV and of HIV DNA in these patients with para-

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doxic responses, however, have not been previously analyzed. The following studies were undertaken to investigate the factors that may contribute to the dissociation of immunologic and virologic responses to HAART.

METHODS

Study Subjects

Flow cytometric quantification of PBMC was performed as described (14). The PR all had $CD4^+$ PBMC counts >135 cells/μl and included 7 individuals with stable $CD4^+$ PBMC counts (<20 cells/μl) and 4 others with increasing $CD4^+$ PBMC counts (>50 cells/μl) during an average follow-up time of 9 ± 3 months. The CF included 9 people with persistently low $CD4^+$ PBMC counts (<60 cells/μl) and 4 others with decreasing $CD4^+$ PBMC counts (>85 cells/μl) during an average follow-up time of 8 ± 3 months. HAART consisted of combinations of PIs including crixivan, nelfinavir, saquinavir, or ritonavir, and RTIs including zidovudine, lamivudine, stavudine, didanosine, nevirapine, and delavirdine.

Plasma Neopterin, β_2-Microglobulin, and HIV RNA Quantification

Enzyme immunoassays were used to quantify plasma concentrations of neopterin (ICN, Costa Mesa, CA, U.S.A.) and β_2M (R&D Systems, Minneapolis, MN, U.S.A.). Plasma HIV RNA concentrations were determined using the Amplicor HIV-1 Monitor test (15) (Roche Diagnostic Systems, Branchburg, NJ, U.S.A.) or by bDNA assays (16) (Chiron, Emeryville, CA, U.S.A.).

Quantification of HIV DNA and Infectious HIV

For quantification of HIV DNA, PBMC were purified from whole blood using Lymphocyte Separation Medium (ICN). PBMC were then lysed in a buffer containing proteinase K and the DNA quantified using a Hoechst dye. Lysates were coamplified for 30 cycles with an internal DNA quantification standard in a prototype assay that uses Amplicor HIV-1 Monitor v1.5 primers SK145-SKCC1B (17,18). Amplified products were quantified in microwell plates using the Amplicor HIV-1 Monitor format. This assay has been shown to yield highly reproducible results and HIV DNA levels determined by this method have been shown to be significantly correlated with plasma HIV RNA levels (19). Infectious units (IU) of HIV in plasma or PBMC were quantified by limiting dilution cultures as described (20,21). Briefly, fivefold dilutions of plasma or PBMC were cultured for 21 days with phytohemagglutinin (PHA)-stimulated PBMC from HIV-seronegative blood bank donors in the presence of T Cell Growth Factor (Cellular Products, Inc., Buffalo, NY, U.S.A.). At the end of 21 days, culture supernatants were analyzed for HIV p24 antigen by enzyme immunoassay (SAIC, Frederick, MD, U.S.A.). Supernatants were scored as either positive (>250 pg/ml) or negative for HIV p24 antigen and the tissue culture infectious dose 50% endpoint ($TCID_{50}$) was calculated as previously described (22). The IU per ml of plasma or per million total PBMC (IUPM) are expressed as the reciprocal of the $TCID_{50}$. The IUPM $CD4^+$ PBMC were calculated by dividing the reciprocal of the $TCID_{50}$ by the percentage of the patient's PBMC expressing CD4.

Statistical Analysis

Levels of PBMC and soluble markers of immune activation are reported as the arithmetic means (X) ± standard error of the mean (SEM). Levels of HIV RNA, HIV DNA, and infectious HIV are reported as geometric means (X) ÷ SEM. Statistical comparisons of PR and CF were performed using Student's, Mann-Whitney, Fisher's exact, or Spearman's tests.

RESULTS

Antiretroviral drug use and levels of $CD4^+$ PBMC and HIV RNA in plasma of one PR are shown in Figure 1. After initiating HAART, this patient had a dramatic increase in $CD4^+$ PBMC counts from <20 cells/μl to >250 cells/μl over a period of 14 months. During this period, his $CD8^+$ PBMC count decreased from 1007 to 847 cells/μl. Except for one brief period after switching therapy to didanosine, nelfinavir, and delavirdine, his plasma HIV RNA concentrations remained between 54,300 and 562,200 copies/ml. After again switching HAART drugs to high doses (3600 mg daily) of saquinavir soft gel capsules, low doses of ritonavir (800 mg daily) plus 80 mg/day stavudine (genotyping showed no mutation at position 75 of the pol gene), a dramatic decrease occurred in plasma HIV RNA levels to 1203 copies/ml and an additional increase in $CD4^+$ PBMC count to 360 cells/μl. In samples taken before this change of therapy when plasma HIV RNA levels were 181,300 copies/ml, we were unable to culture virus from his plasma and found low levels of infectious cell-associated HIV (16.2 IU per million PBMC and 75 IU per million $CD4^+$ PBMC). The levels of this patient's HIV DNA at this time were 5,893 per million total PBMC and 27,408 copies per million $CD4^+$ PBMC. Given that levels of plasma HIV RNA and $CD4^+$ PBMC are usually inversely related, the high levels of HIV RNA and stable or increasing $CD4^+$ PBMC levels in this patient prompted us to compare additional PR with complete failures who demonstrated no significant virologic or immunologic responses to HAART.

In PR (n = 11) and CF (n = 13) at the beginning of the follow-up period, no significant differences were found in the level of $CD4^+$ (PR = 189 ± 56; CF = 88 ± 34) or $CD8^+$ (PR = 1026 ± 183; CF = 866 ± 178) PBMC. Table 1 shows that the mean $CD4^+$ PBMC levels at the end of the follow-up period were significantly higher ($p < .001$) in PR (258 cells/μl) than in CF (45 cells/μl). The PR showed a significant gain in $CD4^+$ PBMC (ΔCD4 = +69) in contrast to CF who lost an average of 43 $CD4^+$ PBMC during the follow-up period

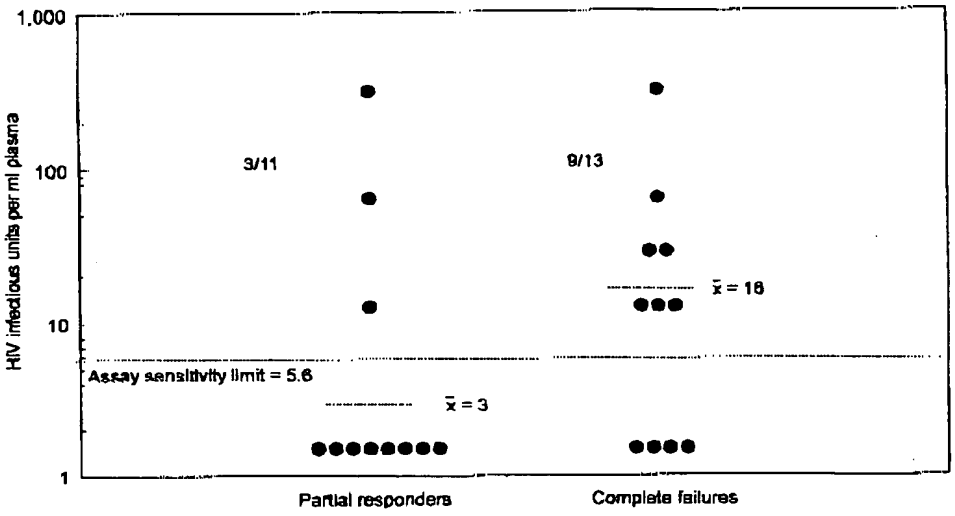

FIG. 3. Levels of infectious HIV in plasma from partial responders and complete failures.

tions make it difficult to compare IUPM values directly, the study demonstrated that differences of this magnitude (i.e., fivefold to 10-fold) were associated with about a twofold increase in the relative risk of AIDS during almost 4 years of observation. In this study, we observed a differential of 112 $CD4^+$ cells per μl (69 cell [27%] increase in PR and 43 cell [96%] decrease in CF) during an average of only 8 months of observation.

A direct causal relationship between increases in $CD4^+$ lymphocyte levels in peripheral blood and reduction of plasma HIV RNA levels resulting from decreased killing of $CD4^+$ cells by HIV as a result of HAART has been suggested (29,30). Evidence of increases in both $CD4^+$ and $CD8^+$ PBMC by HAART as a result of both lymphocyte redistribution from lymph nodes to peripheral blood and lymphocyte proliferation (31), reports of individuals with discordant immunologic and virologic responses to HAART (6–11), and a critical reevaluation of the viral dynamic models (32) challenge this simple model.

Although the PR in our study were observed for an average of 8 months, the observed elevation in $CD8^+$ cells is consistent with the possibility that their $CD4^+$ PBMC levels were influenced at least in part by prolonged redistribution from lymph nodes to peripheral blood. It has been suggested that HAART may cause reduced destruction or increased regeneration of both $CD4^+$ and $CD8^+$ lymphocytes (33) but it is not clear

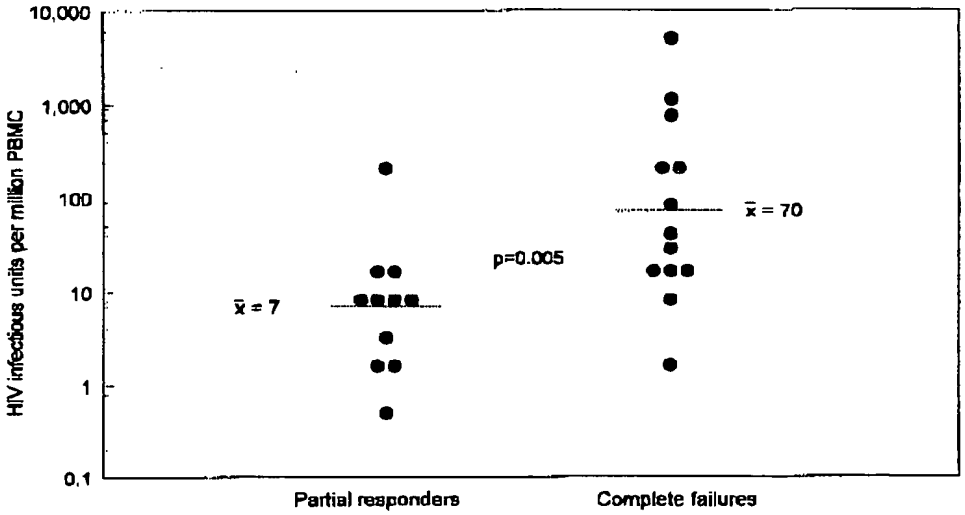

FIG. 4. Levels of infectious HIV in peripheral blood mononuclear cells from partial responders and complete failures.

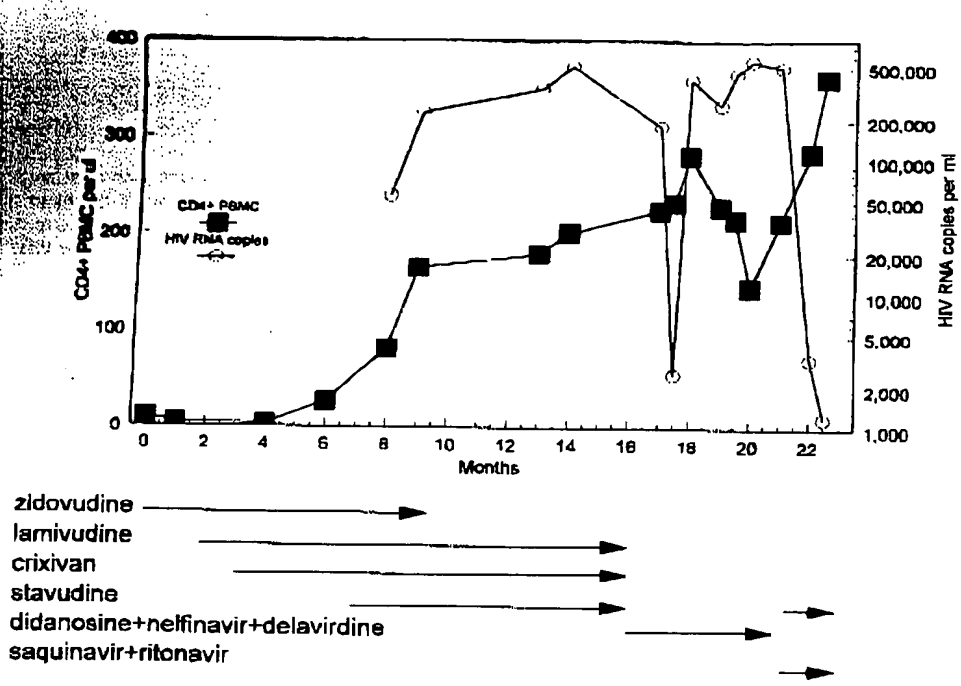

FIG. 1. Plasma HIV RNA, CD4+ peripheral blood mononuclear cells, and antiretroviral drug usage in a partial responder.

of 8 to 9 months ($p < .001$). Levels of $CD8^+$ PBMC were also significantly higher in PR than in CF ($n < .001$). The change in $CD8^+$ PBMC levels ($\Delta CD8$) was also significantly different between PR and CF, increasing by an average of 274 $CD8^+$ PBMC/µl in PR and decreasing by an average of 309 $CD8^+$ PBMC/µl in CF ($n < .001$) over 8 to 9 months of follow-up. Plasma markers of immune activation, β_2M, and neopterin were measured in PR and CF only at the end of the follow-up period and were high in both groups but were not significantly different (Table 1).

Plasma and PBMC were collected at the end of the follow-up period and analyzed quantitatively for HIV RNA, HIV DNA, or IU of HIV (Table 1). The levels of

TABLE 1. *Levels of $CD4^+$ and $CD8^+$ peripheral blood mononuclear cells (PBMC), plasma immune activation markers, and HIV in partial responders and complete failures to highly active antiretroviral therapy (HAART)*

	Partial responders	Complete failures	Significance (p value)
	($n = 11$)	($n = 13$)	
$CD4^+$ PBMC count (cells/µl)[a]	258 ± 47	45 ± 17	<.001
$\Delta CD4^+$ PBMC count (cells/µl)[b]	+69 ± 23	−43 ± 19	<.001
$CD8^+$ PBMC count (cells/µl)[a]	1,300 ± 157	556 ± 91	<.001
$\Delta CD8^+$ PBMC count (cells/µl)[b]	+274 ± 150	−309 ± 116	<.001
β_2-Microglobulin (µg/ml)[a]	6.8 ± 0.4	7.2 ± 0.1	NS
Neopterin (ng/ml)[a]	13.5 ± 2.4	13.9 ± 1.3	NS
Plasma HIV RNA/copies/ml[a] ($\log_{10}$ ± standard error of the mean (SEM)	101,215 (5.0 ± 0.1)	192,581 (5.3 ± 0.1)	.045
HIV DNA copies/10^6 PBMC[a] ($\log_{10}$ ± SEM)	4,243 (3.6 ± 0.1)	4,634 (3.7 ± 0.2)	NS
HIV DNA copies/10^6 $CD4^+$ PBMC[a] ($\log_{10}$ ± SEM)	26,914 (4.4 ± 0.1)	144,160 (5.2 ± 0.2)	.007
HIV DNA copies/ml[a] ($\log_{10}$)	6,944 (3.8)	6,487 (3.8)	NS
Infectious units HIV/ml plasma[a] ($\log_{10}$ ± SEM)	3 (0.1 ± 0.3)	16 (1.2 ± 0.3)	NS
Infectious HIV units/10^6 PBMC[a] ($\log_{10}$ ± SEM)	7 (0.8 ± 0.2)	70 (1.8 ± 0.3)	.005
Infectious HIV units/10^6 CD4 PBMC[a] ($\log_{10}$ ± SEM)	42 (1.6 ± 0.2)	2,165 (3.3 ± 0.3)	<.001
$CD4^+$ IU/ml[a] ($\log_{10}$)	10.8 (1.0)	97.4 (2.0)	<.001

[a] At end of follow-up.
[b] Changes during follow-up.
NS, not significant ($p > .05$). PBMC, β2-microglobulin, and neopterin levels are reported by arithmetic means ± SEM. HIV levels are reported as geometric means and as $\log_{10}$ ± SEM. Levels of $CD4^+$ and $CD8^+$ peripheral blood leukocytes, plasma immune activation markers, HIV RNA, proviral HIV DNA, and infectious units of HIV were quantified as described in Methods.

plasma HIV RNA were only slightly lower (0.3 $\log_{10}$) in PR as compared to CF ($p = .045$). Both groups also showed substantial levels of HIV DNA in their PBMC but the mean levels were not significantly different. Because the CF had much lower levels of CD4$^+$ PBMC than PR the levels of HIV DNA per million CD4$^+$ PBMC were significantly higher ($p = .007$) in the CF than in the PR (Fig. 2). The PR and CF both had high plasma HIV RNA levels but only a very small fraction of this HIV RNA represented HIV particles that were capable of replicating in tissue culture. Although the mean levels of IU of HIV in plasma were not significantly different between PR (3 IU/ml) and CF (16 IU/ml), HIV could be cultured from only 3 (27%) of 11 PR in comparison to 9 (69%) of 13 CF ($p = .03$) (Fig. 3). The ratio of IU to HIV RNA in plasma was 1:12,036 in CF and 1:33,378 in PR, similar to reports in untreated individuals (23,24). When results of CF and PR were combined, the HIV IU/ml of plasma showed a significant correlation with the $\log_{10}$ HIV RNA copy number in plasma ($r = 0.625$; $p < .001$).

Figure 4 shows that the PR had significantly lower levels than CF of IUPM HIV per million total PBMC mononuclear cells (7 versus 70; $p = .005$). When results of CF and PR were combined, the IUPM and IU/10^6 CD4$^+$ PBMC both showed significant correlations with the $\log_{10}$ HIV RNA copy level in plasma ($r = 0.4$; $p < .05$). In that the PR had much higher levels than CF of CD4$^+$ PBMC, the mean levels of IUPM CD4$^+$ PBMC was more than 50-fold lower in PR than CF (42 versus 2,165; $p < .001$). When corrected for CD4 count, the PR had about 10-fold fewer IU per ml of blood than CF (10.8 versus 97.4, respectively).

DISCUSSION

These results demonstrate that, despite a similar number of HIV-infected cells per ml, CF have both a higher proportion of HIV-infected CD4$^+$ cells and a higher proportion of those that are able to initiate new virus production in vitro. The observed differences between PR and CF in total viral RNA and "infectious" RNA per ml were consistent with this finding but smaller than might be expected, given a 10-fold difference in "productively infected" CD4$^+$ cells per ml.

Because PI are thought to function primarily by rendering newly produced virus noninfectious (25), this decreased infectivity in PR versus CF may be due to a limited virologic response in HAART that was not evident in the measurement of viral RNA alone. It has been suggested that PI may act at more than one step in retroviral replication (26) and differential effects of PI and/or RTI in PR and CF may also contribute to these differences in infectivity. Faye et al. have reported data consistent with our observations and suggest decreased viral fitness is associated with a gain in CD4$^+$ PBMC in patients with discordant CD4 and plasma HIV RNA responses to PI therapy (27).

Although observed differences in infectivity may contribute to the improved immunologic responses seen in PR, the critical question is: are these differences large enough to account for the degree of stability and/or increase in CD4 counts in the PR group? We observed a 10-fold difference in IUPM PBMC between PR and CF (7 versus 70 IU per million). A recent study has evaluated the relationship between cell-associated infectious HIV-1 and progression to AIDS or death in untreated individuals (28). Although differences in culture condi-

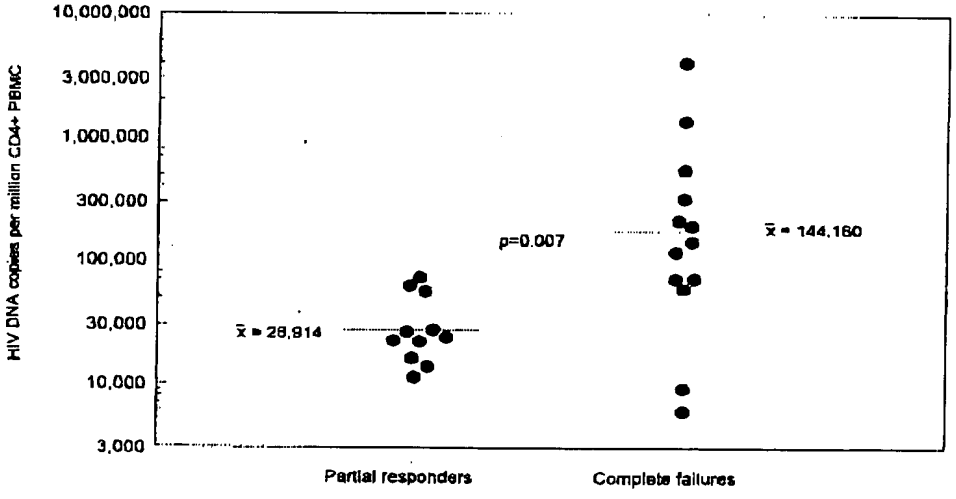

FIG. 2. HIV DNA Levels in CD4$^+$ peripheral blood mononuclear cells from partial responders and complete failures.

whether either or both of these mechanisms may be occurring in PR.

A recent study suggested that the previous rate of CD4 depletion and long-term viral load reduction played an important role in discordant immunologic and virologic responses in the first few months after initiation of HAART (10). Levels of infectious HIV and HIV DNA and their influence on immunologic and virologic responses to HAART were not, however, evaluated in that study. Although data regarding $CD4^+$ depletion rates before initiation of HAART are not available for our study subjects, the duration of the discordant responses for more than 1 year in some of the PR make this an unlikely explanation for our observations.

Because peripheral blood represents only ≤ 2% of the total lymphocyte pool, even small effects of PI and/or RTI on lymphocytes in lymph nodes may influence our observations of PBMC in PR. It has been suggested that increases in peripheral blood shortly after the initiation of HAART may be primarily the result of lymphocyte redistribution from the lymph nodes to peripheral blood (31). Other recent studies (34–37) show that HAART reverses some of the immunoactivation that is associated with HIV infection and that this may be independent of its antiviral activity (36). This possibility offers an appealing explanation for the findings reported here.

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